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First record of ranavirus (*Ranavirus* sp.) in Siberia, Russia

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Abstract

Ranaviruses are a group of double-strand DNA viruses that infect fish, amphibians and reptiles. These viruses are responsible for mass fish and amphibian mortality events worldwide, both in the wild and at fish and amphibian farms. The number of detected epizootics has grown significantly in recent years. In Eastern Europe and Northern Asia, including Russia, very few ranavirus monitoring studies have been conducted, in contrast with Western Europe and America. In the present work, we used a qPCR assay to survey for the first time the amphibian populations of West Siberia (Russia) for the presence of ranaviruses. In total, we studied 252 tissue samples from six amphibian species, collected across West Siberia from the south to the Arctic regions. We report a single infected sample: a common toad (*Bufo bufo*) captured near Tyumen city. The phylogenetic analysis showed that the detected virus strain belongs to the CMTV lineage. This is only the second observation of *Ranavirus* in Russia.

Key Words

amphibian pathogens, *Bufo*, emergent diseases, qPCR, toad, West Siberia

Introduction

Ranaviruses are a genus of double-strand DNA viruses of the family Iridoviridae, which infect ectothermic vertebrates. The ranaviral infection may lead to significant disease and mortality rate in fish and amphibians, both in the wild and in aquaculture (Price et al. 2014; George et al. 2015; Kwon et al. 2017; Deng et al. 2020). Along with the chytrid fungi, the ranaviruses are considered as one of the emerging amphibian and fish infections, since the numbers of detected mortality events rose significantly in the last decades (Duffus et al. 2015). There are several explanations for this rise. First, ranaviruses are likely spread alongside the global fish and amphibian trade (Brunner et al. 2019). Second, infection outbreaks may be caused by native viruses and triggered by climate change, pollution and other stress-inducing factors (Price et al. 2019). Third, the rise in detected cases may be due to increased awareness and better detection methods (Miaud et al. 2019). Most probably, all these mechanisms are involved, with unknown relative inputs. The detection of ranaviruses in remote and sparsely populated areas shows that their global distribution is likely natural (D'Aoust-Messier et al. 2015).

There are few monitoring studies of the ranavirus infections in Eastern Europe and Northern Asia. Their presence has been detected in Poland and Hungary, both in fish and amphibian hosts (Borzym et al. 2013, 2020; Juhász et al. 2013; Vörös et al. 2020; Palomar et al. 2021). In Russia, the only previous study detected the presence of a ranavirus near Moscow (Reshetnikov et al. 2014). Therefore, these pathogens are possibly widely distributed in Russia, similarly to Hungary and Poland, albeit almost completely unstudied. In this work, we conducted a survey to detect the presence of ranaviruses in West Siberia. This region is distant from all the known locations of the ranavirus presence, therefore studying it fills an important gap in the knowledge of the ranavirus distribution.

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Materials and methods

During the 2020 and 2021 field seasons, we collected 252 tissue samples from the following species: *Bufo bufo* (136 samples), *Bufotes* cf. *viridis* (5 samples), *Rana arvalis* (91 samples), *Rana amurensis* (2 samples), *Lissotriton vulgaris* (8 samples), *Salamandrella keyserlingii* (10 samples). We used toe clips for the adult anurans and tail clips for the anuran tadpoles and for the caudates, and the animals were subsequently released at the place of capture. The samples were collected across West Siberia, Russia (Fig. 1A) in 14 localities. For the detailed list of samples and localities see Suppl. material 1.

DNA was extracted either using the QIAGEN QIAamp DNA mini kit (Germany) (37 samples), by the standard phenol-chloroform technique (Sambrook and Russell 2006) (202 samples), or by the salting-out method (Aljanabi and Martinez 1997) (13 samples). To detect the presence of the ranavirus, we used the qPCR assay developed by Leung et al. (2017). This method is a duplex PCR utilizing TaqMan probes, with one set of primers specific to a fragment of the viral MCP gene, and another set of primers specific to the reference vertebrate ultraconservative non-coding single-copy element EBF3N. The amplification of EBF3N was used to ensure the DNA quality and successful course of PCR. Each sample, positive and negative control were run in two duplicates. As positive control, we used the DNA extracted from the liver of a *Pelophylax esculentus* specimen that died due to the ranaviral infection, provided by Dr. Vojtech Baláž (University of Veterinary Sciences Brno, Czech Republic).

The PCR was prepared using the BioMaster HS-qPCR master mix (Biolabmix, Novosibirsk, Russia). The oligonucleotides were prepared by Evrogen (Moscow, Russia). The total reaction volume was 15 µl, the DNA solution volume used in each reaction was 1 µl. The primer concentrations were 0.5 pM, and the probe concentrations were 0.25 pM. The PCR was run for 50 cycles and the annealing temperature was 60 °C. Amplification was carried out using the Roche LightCycler 96 or BioRad CFX96 real-time PCR systems, and the amplification curves were analyzed using the respective official software. The samples were considered positive if a robust sigmoidal amplification curve was present in at least one of the two duplicates.

To amplify longer fragments of the MCP gene for sequencing, we developed the following primers using the NCBI Primer Blast service (https://www.ncbi.nlm.nih.gov/tools/primer-blast/): RV1_F 5`-CTGGTGTACGAAAACACCACAAG-3`, RV1_R 5`-CGTTCATGATGCGGATAATGTTGT-3`, RV2_F 5`-ATCAGGATAACAGTCAAGCTGAGG-3`, RV289_R 5`-TGTGTGACGTTCTGCACCATAAA-3`. To design the primers, we obtained a consensus MCP sequence based on all complete *Ranavirus* MCP sequences available in GenBank, and used it as a template. To avoid non-specific annealing to the host genome, we performed a specificity check against the genome of *Bufo bufo* (assembly aBufBuf1.1). PCR with these primers was conducted using the BioMaster HS-Taq PCR-Color master mix (Biolabmix, Novosibirsk, Russia) with the following protocol: 96 °C for 5", 40 cycles of amplification (96 °C for 15', 60 °C for 30', 72 °C for 30'), and 72 °C for 5". The obtained products were analyzed using the 1.5% agarose gel, purified with the PCR cleanup kit (BioSilica, Novosibirsk, Russia) and Sanger sequenced bidirectionally at Evrogen (Moscow, Russia).

The forward and reverse sequences were analyzed and merged using MEGA 11 (Tamura et al. 2021). For

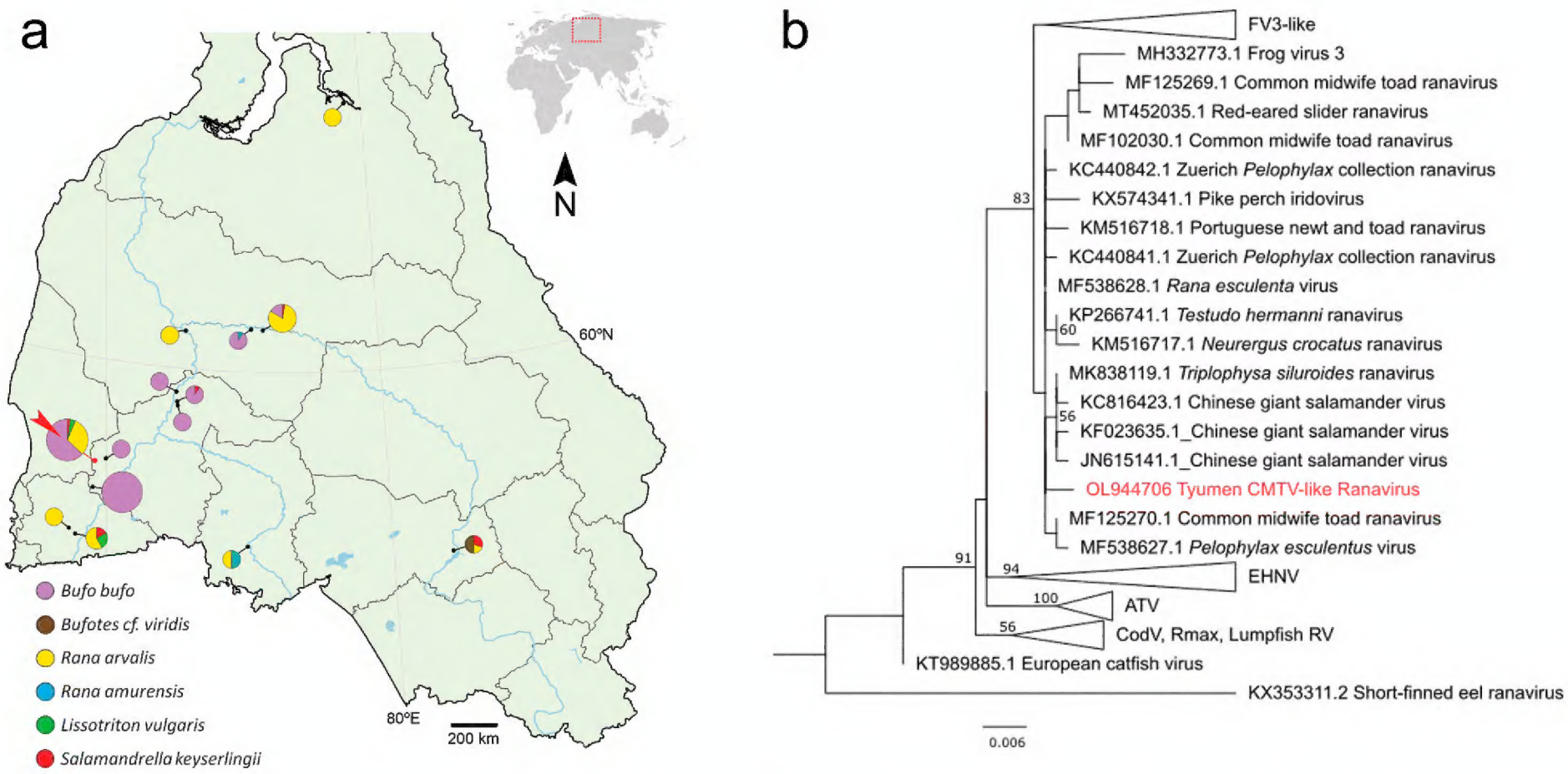

Figure 1. A. The map showing the geographical distribution of the screened samples. The circle size is proportional to the sample size. The red arrowhead points to the locality where the ranavirus was detected; **B.** The ML phylogenetic tree showing the position of the identified ranavirus strain (in red). FV3-like: frog virus 3 like; EHNV: epizootic haematopoietic necrosis virus; ATV: *Ambystoma tigrinum* virus; CodV: cod iridovirus; Rmax: *Ranavirus maximus*; Lumpfish RV: lumpfish ranavirus.

the phylogenetic analysis, the sequences from two primer pairs (total length 554 bp) were assembled in one sequence (703 bp including gap) and aligned to 55 *Ranavirus* MCP fragment sequences mined from the GenBank using ClustalW in MEGA11. The phylogenetic analysis was conducted using the Maximum Likelihood (ML) method in IQ-TREE 2 (Minh et al. 2020) with the K3Pu+F+G4 substitution model, selected by ModelFinder (Kalyaanamoorthy et al. 2017), with 1000 bootstrap replicates.

Results

A single positive sample of common toad (*B. bufo*) was found in a water body near the city of Tyumen (57°11.08'N, 65°10.57'E). All other samples did not show amplification of the *Ranavirus* DNA. All tested samples showed amplification of the reference EBF3N locus. To ensure that the positive result is not due to contamination, we extracted the DNA from the same tissue sample again, in another laboratory, with equipment and reagents never exposed to ranaviral DNA. The first DNA sample was extracted using the phenol-chloroform method, and the second was extracted using the QIAGEN kit. Then we performed a PCR with freshly unpacked and prepared reagents, and the positive result was confirmed.

Only circa 50% of the replicates showed the amplification of ranaviral DNA (mean Ct = 37.5±0.8 (36.4–38.5), n = 5). This confirms the validity of our finding but shows very low infection load in the toe tissue of the sampled toad.

The newly designed primers for the larger fragments of the MCP gene have no matches in the *B. bufo* genome and delivered the desired fragments as the only products. The sequence of two fragments of the MCP gene (703 bp in total, with ambiguous sites insert of 149 bp to connect the fragments) was deposited in GenBank (OL944706). The ML phylogenetic analysis showed that the detected strain of *Ranavirus* belongs to a new variant within the CMTV-like clade (Fig. 1B). The p-distance between the detected ranavirus and the closest relative from GenBank was 0,36% (two substitutions).

Discussion

Our work presents the first case of detection of the ranavirus infection in Siberia, and the second case in Russia (Reshetnikov et al. 2014). Possibly, the low prevalence of ranaviruses in our study is explained by the cold local climate with relatively short periods of amphibian activity, which impairs the spreading of the viruses. In fact, ranaviruses were reported from northeastern Canada in the areas with climate even colder than in southern Siberia (D'Aoust-Messier et al. 2015). However, there are currently too few studies of ranaviruses in the high latitude areas, and it is possible that their abundance decreases to the north. It is known that these viruses require relatively warm temperatures for activity. In Great Britain, it was found that warmer temperatures are associated with higher occurrence and severity of ranaviral infections in *R. temporaria* (Price et al. 2019).

The Ct values in the infected common toad in our study are similar with those reported for other similar samples studied using the same protocol (Vörös et al. 2020; Palomar et al. 2021). Ranaviruses primarily infect internal organs, therefore low viral loads are common for toe and tail samples collected from clinically healthy animals (Wynne 2019).

Initially, to amplify the fragments of the MCP gene for sequencing, we attempted to use the primers developed by Holopainen et al. (2009). However, in our sample they returned multiple non-specific amplicons from the host DNA and no specific amplicons. In contrast, in the positive control sample with higher concentration of the ranaviral DNA they returned single amplicons that corresponded to the predicted length. The inability to amplify long MCP fragments from positive samples with low infection load was also reported by Wynne (2019). The newly designed primers for the fragments of the *Ranavirus* MCP gene are highly specific and may be used to amplify the MCP gene fragments from the infected samples of this and possibly related species with low viral loads. The identified CMTV-like strain of *Ranavirus* is related to some other European CMTV strains, like the PNTRV and the Zuerich *Pelophylax* virus. It is also close to the *Andrias davidianus* ranaviruses from China. Interestingly, the ranavirus from the lake Glubokoe in the Moscow region belongs to the FV3 clade (Reshetnikov et al. 2014). Thus, our work is the first detection of a CMTV-like ranavirus in Russia.

The water body where the infected toad was found represents an old artificial pond in the forest created as a water reservoir to use in case of a forest fire. The cohabiting amphibian and fish species include *R. arvalis*, *S. keyserlingii*, *L. vulgaris*, *Carassius auratus*, *Rhynchocypris percnurus*. No animals with clinical signs of ranavirosis were detected in the pond. The pond is used by the locals for recreation and fishing, and the fish were possibly introduced there by anthropogenic means. This implies that the ranaviruses, although rare in Siberia, may still pose a threat for the local wild populations and aquaculture, and be artificially spread between water bodies by local fishermen, together with the natural spreading with migrating amphibians.

Conclusions

The current study reports the second finding of a ranavirus in Russia, and the first in Siberia. We found that ranaviruses are rare in the studied regions of Siberia, since only one infected toad was found among the screened amphibians. The detected virus strain belongs to the CMTV lineage, which is the first record of this lineage in Russia. More extensive sampling of Siberian amphibians and fish is required to elucidate the spread and diversity of ranaviruses in the region in more detail, and special attention should be paid to invasive species.

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Supplementary material 1

Geographic distribution, species and sample sizes of the amphibians screened for the ranavirus infection

Authors: Artem P. Lisachov, Lada S. Lisachova, Evgeniy Simonov

Data type: xls file

Explanation note: The table lists all sampled localities with geographic coordinates, and the numbers of screened specimens of each species in each locality.

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